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Tumor cell alpha-*N*-acetylgalactosaminidase activity and its involvement in GcMAF-related macrophage activation

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Abstract

Alpha-*N*-acetyl galactosaminidase (alpha-NaGalase) has been reported to accumulate in serum of cancer patients and be responsible for deglycosylation of Gc protein, which is a precursor of GcMAF-mediated macrophage activation cascade, finally leading to immunosuppression in advanced cancer patients. We studied the biochemical characterization of alpha-NaGalase from several human tumor cell lines. We also examined its effect on the potency of GcMAF to activate mouse peritoneal macrophage to produce superoxide in GcMAF-mediated macrophage activation cascade. The specific activity of alpha-NaGalases from human colon tumor cell line HCT116, human hepatoma cell line HepG2, and normal human liver cells (Chang liver cell line) were evaluated using two types of substrates; GalNAc-alpha-PNP (exo-type substrate) and Gal-beta-GalNAc-alpha-PNP (endo-type substrate). Tumor-derived alpha-NaGalase having higher activity than normal alpha-NaGalase, had higher substrate specificity to the exo-type substrate than to the endo-type substrate, and still maintained its activity at pH 7. GcMAF enhance superoxide production in mouse macrophage, and pre-treatment of GcMAF with tumor cell lysate reduce the activity. We conclude that tumor-derived alpha-NaGalase is different in biochemical characterization compared to normal alpha-NaGalase from normal Chang liver cells. In addition, tumor cell-derived alpha-NaGalase decreases the potency of GcMAF on macrophage activation.

Keywords: Alpha-*N*-acetylgalactosaminidase; Gal-beta-GalNAc-alpha-PNP; GalNAc-alpha-PNP; GcMAF-mediated macrophage activation cascade; Gc protein-derived macrophage activating factor (GcMAF); Mouse peritoneal macrophage; Superoxide; Tumor cells

1. Introduction

Alpha-*N*-acetylgalactosaminidase (alpha-NaGalase, EC 3.2.1.49) is a lysosomal enzyme that hydrolyzes the *O*-glycosidic linkage between terminal alpha-*N*-acetylgalactosamine moiety and serine or threonine in mucin-type glycoprotein (Zhu et al., 1998). Human alpha-NaGalase is encoded by a gene localized to chromosome 22q13→qter (Wang and Desnick, 1991). A full-length molecular cloning of human alpha-NaGalase cDNA has been reported to consist of a 46-kDa molecular weight protein and shows striking homology with human alpha-galactosidase A and yeast alpha-galactosidase (Tsuji et al., 1989). A deficiency of alpha-NaGalase activity in humans may cause severe clinical disorders such as Schindler's disease (Schindler et al., 1989; Wang et al., 1990). An endo-alpha-NaGalase has been isolated from *Diplococcus pneumoniae* with a molecular weight of approximately 160 kDa, determined by gel filtration, an optimum pH of 7.6 and an isoelectric point in the range of pH 8–9 (Umemoto et al., 1977). Although the endo-type alpha-NaGalase has

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been reported in bacteria, only the exo-type of alpha-NaGalase has been reported in humans.

Inflammation results in macrophage activation leading to immune development (Tedla et al., 1999). Serum vitamin D_3-binding protein (Gc protein), a 51.2 kDa polymorphic protein of the $alpha_2$-macroglobulin fraction of human plasma, can be converted by an inducible beta-galactosidase of B cells and sialidase of T cells to a potent macrophage activating factor (MAF), a protein with *N*-acetylgalactosamine as the remaining sugar moiety (Yamamoto et al., 1996). Thus, the inflammation-primed macrophage activation process is the major macrophage activation cascade, which requires participation of B and T lymphocytes and Gc protein. Interestingly, Kanan et al. recently reported non-inducible generation of GcMAF, showing that GcMAF was generated even in the absence of a mediator of inflammation (Kanan et al., 2000).

Meanwhile, alpha-NaGalase, has been reported to accumulate in cancer patients and be responsible for deglycosylation of Gc protein, finally leading to immunosuppression in advanced cancer patients (Yamamoto et al., 1996, 1997a). However, this postulate lacks evidence for tumor-derived alpha-NaGalase activity on GcMAF generation and its influence in GcMAF-mediated macrophage activation cascade. We also checked any possibility of alpha-NaGalase from human tumor cell lines being an endo-type glycosidase, as the hypothesis scheme of alpha-NaGalase activity indicated the possibility of tumor-derived alpha-NaGalase to be an endo-type glycosidase. Because GcMAF is a promising immunomodulator for future cancer immunotherapy we study the biochemical characterization of alpha-NaGalases from 2 types of human tumor cell lines; human hepatoma cell lines HepG2, a well-differentiated cell line (Chang et al., 1993) and human colon tumor cell lines HCT116, which have been reported to be induced differentiation and apoptosis with cytochalasin and brefeldin A derivatives (Nagasawa et al., 2000; Zhu et al., 2000). We compared alpha-NaGalase activity from these cell lines to normal human liver cells (Chang liver cell line) (Chang, 1954; Fortunati et al., 1993). The effect of alpha-NaGalase on GcMAF-mediated macrophage activation was estimated by superoxide generation assay.

2. Materials and methods

2.1. Materials

The exo-type substrate, *p*-nitrophenyl 2-acetamido-2-deoxy-alpha-D-galactopyranoside was obtained from Funakoshi Co., Japan. Endo-type substrate, *p*-nitrophenyl 2-acetamido-2-deoxy-3-*O*-(beta-D-galactopyranosyl)-alpha-D-galactopyranoside was purchased from Seikagaku Co., Japan. RPMI 1640, McCoy's 5A and Dalbecco's MEM were purchased from GIBCO BRL. Chicken liver derived alpha-*N*-acetylgalactosaminidase, alpha-galactosidase derived from *Aspergillus niger*, Jack Bean derived beta-galactosidase, cytochrome *c* derived from horse heart, phorbol myristate acetate (PMA) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Japan Co., Japan. Endo-type alpha-*N*-acetylgalactosaminidase derived from *Diplococcus pneumoniae* and other chemicals (biochemical grade) were purchased from Wako Pure Chemical Industries Co., Japan.

2.2. Cell culture and sample preparation

Cell lines were cultured in 10% heat inactivated fetal calf serum containing Dalbecco's MEM medium for HepG2 and Chang liver cell line, and McCoy's 5A medium for HCT116 cell line. The cells were harvested using 0.02% EDTA and sonicated using Ultrasonic Disruptor UD-200 TOMY in 3 ml of cold 15 mM Tris–HCl (pH 7.0) in an ice bath. The samples were centrifuged at $11\,000 \times g$ for 15 min to exclude the cell membrane and other husk from the suspension. The supernatant was precipitated by 70% saturated ammonium sulfate and the homogenates were clarified by centrifugation at $11\,000 \times g$ for 15 min, then it was dissolved in 50 mM citrate buffer pH 5.0, 6.0 or 7.0 as indicated in the results. After dialysis with the same buffer the samples were used in the enzyme activity assay.

2.3. Alpha-N-acetyl-galactosaminidase activity assay

The procedure was adapted from methods reported by Yagi et al. (Yagi et al., 1990). The enzyme activity was determined at 37 °C in a reaction mixture of 1 ml of 50 mM citrate buffer containing 5 μM of exo-type or endo-type substrate. The reaction was initiated by addition of 50

μM sample and terminated by 100 μl of 10% trichloroacetic acid. After centrifugation of the reaction mixture, 150 μl of 0.5 M Na_2CO_3 was added to the mixture and after centrifuge at 15 000×*g* for 15 min, the amount of released *p*-nitrophenol was measured spectrophotometrically at 420 nm (U-3010, HITACHI). Specific activity was calculated based on the standard curve of *p*-nitrophenol at 420 nm. Protein concentration was determined by BCA reaction (the Pierce BCA Protein Assay).

2.4. Isolation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were collected from BALB/c mice 4 days after inoculation with 3% thioglycollate. After centrifugation at 1000×*g* at 4 °C for 10 min, the collected macrophages were cultured in 6-well plates with a concentration of $5\sim10\times10^5$ cells/well in 10% FBS/RPMI 1640. Cultured cells were then washed 3 times with PBS(−) to separate adherent macrophages from non-adherent cells (T and B cells). The cultured macrophages were then treated with lipopolysaccharide (LPS) and GcMAF for 24 h, and superoxide generation assay was done as described below.

2.5. Superoxide generation assay

The method was modified from that reported by Johnston et al. (Johnston et al., 1978). Briefly, after drug treatment for the times indicated, the plates were washed twice with PBS(-) and once with Krebs–Ringer phosphate buffer, and 1.5 ml of 80 μM cytochrome *c* in Krebs–Ringer phosphate buffer was added to the plates. PMA was added to the final concentration 100 ng/ml in each well and cultured for 90 min at 37 °C/5% CO_2 in a humidified incubator. The reaction was stopped by means of an ice-bath and the cultured medium was placed in an Eppendorf tube and promptly cleared by centrifugation at 8000×*g*. The optical density of the supernatant was determined spectrometrically at 550 nm with reference at 540 nm (U-2000, HITACHI), using mixtures from plates without cells as blanks. The concentration of reduced cytochrome *c* was determined using the equation $\Delta E_{550\ nm}=2.1\times10^4\ M^{-1}\ cm^{-1}$.

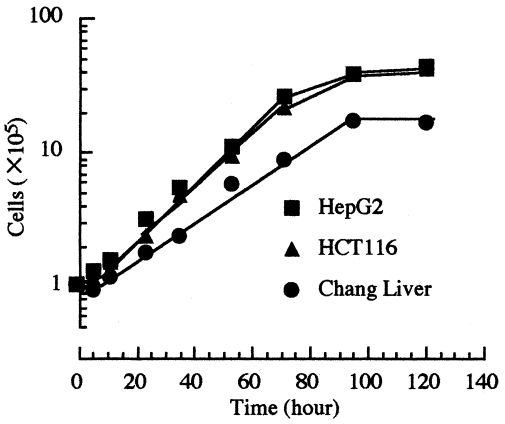


Fig. 1. Growth curves of HepG2, HCT 116 and Chang Liver cell lines.

2.6. GcMAF preparation

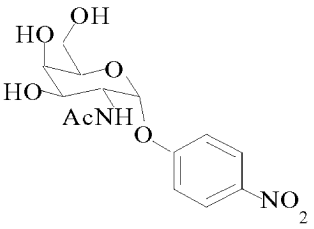
Twenty-five micrograms of group-specific protein (Gc protein) derived from human plasma (Athens Research and Technology Inc.) was dissolved in 100 μl of 50 mM citrate buffer pH 5.0. 0.1 U/2 μl sialidase (Type VI, Sigma Co.), beta-galactosaminidase (Type III, Sigma Co.) was added to the solution and the reaction mixture was incubated at 37 °C for 1 h. The reaction was stopped by means of an ice-bath and the volume was made up to 500 μl.

3. Results

3.1. Alpha-NaGalase activities in human tumor cell lines

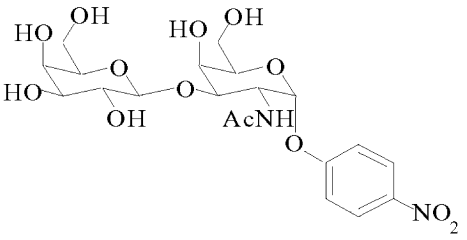
In order to characterize the biochemical activity of tumor-derived alpha-NaGalase, we studied alpha-NaGalase activity from two human tumor cell lines, HCT116 (human colon tumor cell line) and HepG2 (human hepatoma cell line). We compared the alpha-NaGalase activity from these tumor cell lines with alpha-NaGalase from Chang liver cell line, a normal human cell line. As expected, tumor cell lines grow faster compared to the normal cell line (HCT116; Doubling Time (DT) = 15.6 h, HepG2; DT = 14.7 h, Chang liver; DT = 21.5 h) as shown in Fig. 1. Alpha-NaGalase activity was examined using PNP-substrates (as shown in Fig. 2), where GalNAc-alpha-PNP was used as exo-type substrate and Gal-beta-GalNAc-alpha-PNP was used as endo-type substrate, to

(a) Exo-type substrate



GalNAc-alpha-PNP

(b) Endo-type substrate



Gal-beta-GalNAc-alpha-PNP

Fig. 2. Substrates used to determine alpha-NaGalase activity.

determine hydrolysis-type activity of alpha-NaGalase.

We studied the pH dependence of alpha-NaGalase using PNP-alpha-GalNAc at pH 5.0, 6.0 and 7.0. Fig. 3 shows the activity of alpha-NaGalase detected at pH 5.0 for all cells (HepG2: 127.29 ± 14 nmol/mg protein/h; HCT116: 155.95 ± 3 nmol/mg protein/h; Chang liver: 72.63 ± 2 nmol/mg protein/h). Alpha-NaGalase activity was found to decrease with the increase of pH. At pH 7.0, no activity of alpha-NaGalase was detected in Chang liver cell lysate. On the other hand, human tumor cell lines still maintain their activity at pH 7.0 (HepG2: 57.48 ± 19 nmol/mg protein/h, 45% of its activity at pH 5.0; HCT116: 4.83 ± 3 nmol/mg protein/h, 3% of its activity at pH 5.0).

Cell lysate from HepG2 cell line (Fig. 4) was found to hydrolyze exo-type of the substrate (GalNAc-alpha-PNP) but no activity was detected with endo-type of the substrate (Gal-beta-GalNAc-alpha-PNP). Endo-type alpha-NaGalase from *Diplococcus pneumoniae* significantly hydrolyzed Gal-beta-GalNAc-alpha-PNP to release *p*-nitrophenol into the reaction mixture, and no cross activity of the enzyme and substrate was detected in the assay system (Table 1). These observations suggest that alpha-NaGalase from HepG2 tumor cell line is an exo-type enzyme.

3.2. GcMAF-mediated macrophage activation cascade and effect of alpha-NaGalase

We prepared GcMAF as described in materials and methods. Fig. 5 shows that GcMAF (21.44 ± 3 nmol $O_2^- / 10^6$ cells/90 min at 1 ng/ml) was a

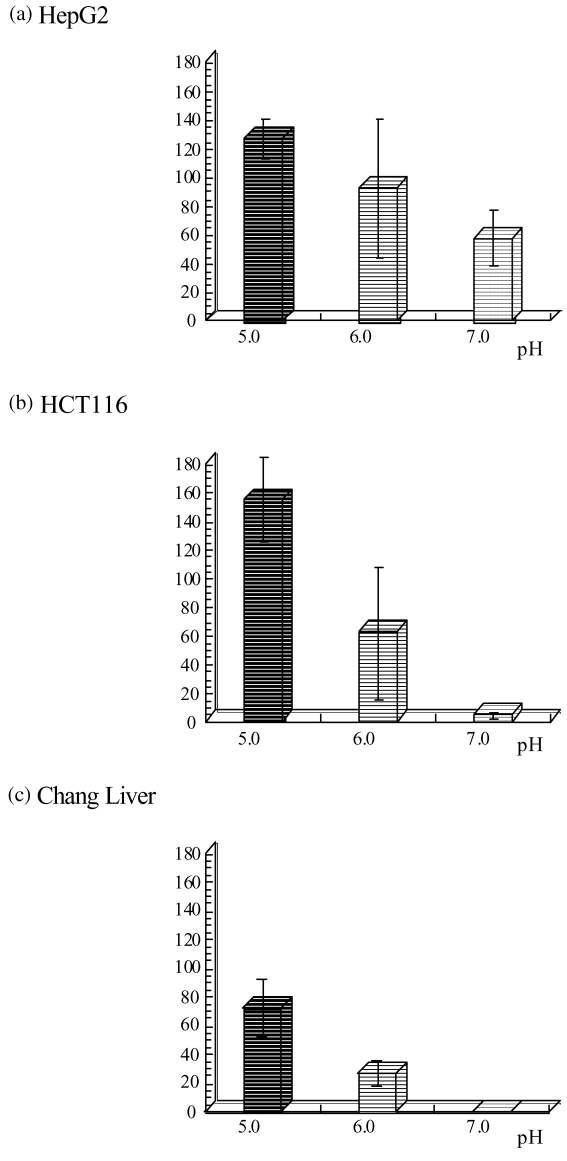


Fig. 3. pH dependence of specific activity of alpha-NaGalase in cell lines. Each value is the mean of three experiments and the error bars represent the S.D.

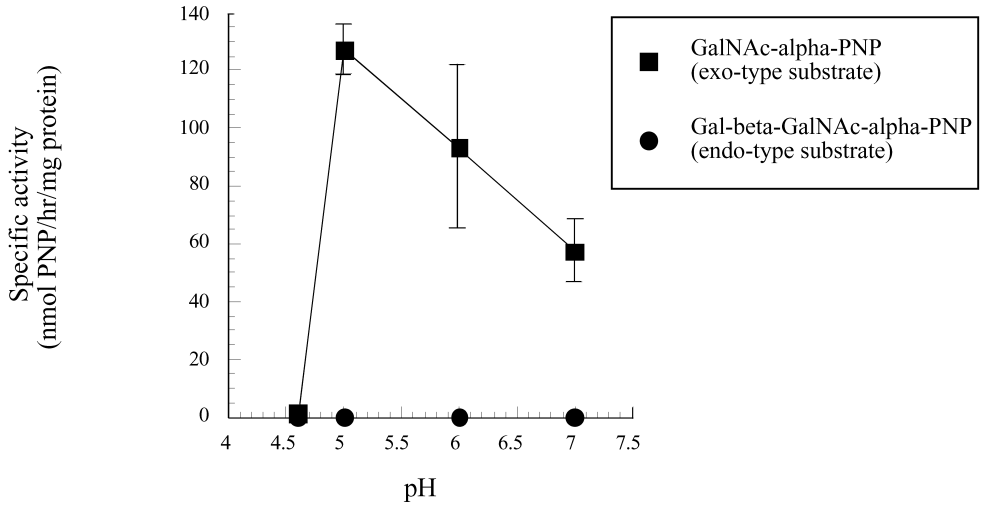


Fig. 4. Hydrolysis type of alpha-NaGalase in HepG2 cell line. Each value is the mean of three experiments and the error bars represent the S.D.

more potent macrophage activator than LPS (28.42 ± 9 nmol $O_2^-/10^6$ cells/90 min at 10 μg/ml). LPS gram-negative bacteria endotoxin and a known macrophage activating agent (Schultz et al., 1978), were used as a positive control in comparing GcMAF activity. Pre-treatment of GcMAF with 0.1 U alpha-NaGalase reduced the macrophage activating potency of GcMAF. Pre-treatment of GcMAF with cell lysate from HepG2 cell line also produce the same observation.

4. Discussion

Deglycosylation of serum vitamin D_3-binding protein (Gc protein) by alpha-NaGalase from tumor tissue has been reported to induce immunosuppression in advanced cancer patients (Yamamoto et al., 1996, 1997b). We studied the biochemical characteristic of alpha-NaGalase from two types of human tumor cell lines (HCT116 and HepG2 cell line) and a normal cell line from human liver (Chang liver cell line). Yamamoto et al. reported a high level of alpha-NaGalase activity in cancer patient serum, and cancer tissue would be the possible origin for alpha-NaGalase in cancer patient serum (Yamamoto et al., 1997a). We found that cell lysate from tumor cell lines showed higher alpha-NaGalase activity compared to the normal Chang liver cell line. Our results support the postulate and also suggest that tumor cells would be the possible origin for alpha-NaGalase in cancer patient serum. Alpha-NaGalase in normal cell is a

Table 1
Substrate specificity of several glycosidases

Enzymes	Specific activity (nmol/h/mU)		
	GalNAc-alpha-PNP	Gal-beta-GalNAc-alpha-PNP	Gal-beta-PNP
endo-alpha-*N*-acetyl-galactosaminidase[a]	$<7.5\times10^{-4}$	18.44	$<7.5\times10^{-4}$
alpha-*N*-acetyl-galactosminidase[b]	0.275	$<7.5\times10^{-4}$	$<7.5\times10^{-4}$
beta-Galactosidase[c]	$<7.5\times10^{-4}$	$<7.5\times10^{-4}$	0.224
alpha-Galactosidase[d]	$<7.5\times10^{-4}$	$<7.5\times10^{-4}$	$<7.5\times10^{-4}$
alpha-*N*-acetyl-galactosaminidase + beta-Galactosidase	ND[e]	$<7.5\times10^{-4}$	ND

[a] EC 3.2.1.97 (*Diplococcus pneumoniae*).
[b] EC 3.2.1.47 (Chicken liver).
[c] EC 3.2.1.23 (Jack beans).
[d] EC 3.2.1.22 (*Aspergillus niger*).
[e] Not determined.

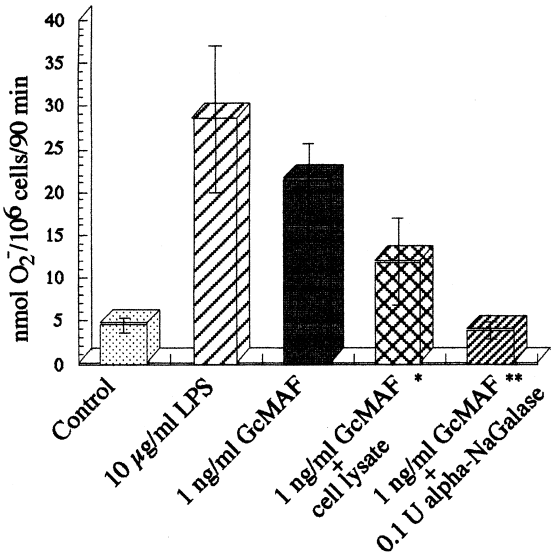


Fig. 5. Effect of alpha-NaGalase in GcMAF-mediated macrophage activation cascade. * 50 μl cell lysate was added during the preparation of GcMAF at pH 5. ** 0.1 U alpha-NaGalase from chicken liver was added during the preparation of GcMAF at pH 5. Each value is the mean of duplicate experiments.

lysosomal enzyme and a factor(s) that leads to the secretion of the enzyme has not been studied yet. The enzymatic regulation of glycosylation in human colonic adenosarcomas was reported to show an altered profile of glycosidases activities in comparison to normal cells (Gil-Martin et al., 1999, 1997). Ioannou et al. reported that overexpression of human lysosomal alpha-galactosidase A in CHO cell lines resulted in their selective secretion, and other lysosomal enzymes such as alpha-NaGalase and acid sphingomyelinase could have the same mechanism (Ioannou et al., 1992). On the other hand, Uematsu et al. reported the possibility of alpha-NaGalase having an oncogenic nature (Uematsu et al., 1999). Our results showed the different character of tumor cell alpha-NaGalase, where the enzyme remains activity at pH 7.0. No activity was detected in normal cell lysate at that pH. Overall findings suggest that tumor cell alpha-NaGalase is different from normal lysosomal alpha-NaGalase.

GcMAF enhanced Fc receptor-mediated phagocytosis in murine peritoneal macrophage by inducing translocation of FcgammaRI and FcgammaRII from intracellular storage compartments to the cell surface, where Gal/GalNAc specific lectin of the macrophage surface membrane may be responsible for GcMAF-mediated macrophage activation (Ono et al., 1995). The core GalNAc moiety for GcMAF-mediated macrophage activation cascade was reported in the third domain of Gc protein (Viau et al., 1983). Ray reported that bacterially expressed recombinant Gc protein had no effect on macrophage activation, because the Gc protein did not contain any sugar moiety (Ray, 1996). Our studies strongly support the hypothesis that core GalNAc moiety is essential for GcMAF-mediated macrophages activation cascade. However, the effect of other endogenous ligands, such as vitamin D_3, in the GcMAF-mediated macrophage activation cascade is still unknown.

Activated macrophages are known to attack and eliminate tumor cells. Yamamoto et al. and Koga et al. reported the possibility of using GcMAF as an immunomodulator for cancer treatment (Koga et al., 1999; Yamamoto, 1996). Our results showed that tumor-derived alpha-NaGalase was an exotype glycosidase, deglycosylates the terminal GalNAc moiety of GcMAF and decrease the potential of GcMAF to activate macrophage. We propose as shown in Fig. 6 that tumor-derived alpha-NaGalase would be active at physiological condition in serum and deglycosylates GcMAF, which finally leads to immunosuppression in advanced cancer patients. Accumulation of alpha-NaGalase from tumor cells into the blood stream would decrease the effectiveness of GcMAF treatment in cancer patients. Therefore, it is necessary to design and develop more specific inhibitors for tumor-derived alpha-NaGalase than for normal alpha-NaGalase (Nishimura et al., 1996).

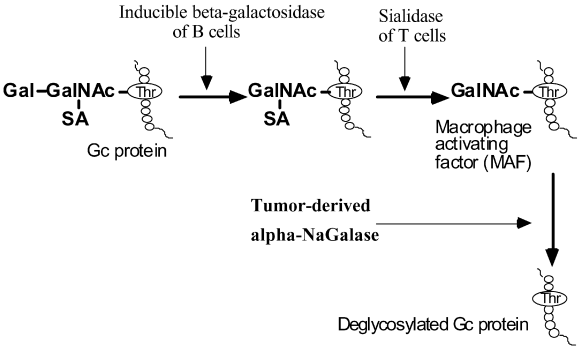


Fig. 6. Proposal for GcMAF deglycosylation scheme in GcMAF-mediated macrophage activation cascade.

Acknowledgments

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